release dysfunction. Cardiac electrical dysfunction in CPVT is remarkably similar to that elicited by digitalis toxicity and a related glycoside, ouabain, has been identified as an endogenous Ca2+-mobilising 'hormone'. Circulating levels of adrenally-derived ouabain are reportedly elevated in heart failure and post-exercise. Here we investigated whether physiologic levels of ouabain could provoke mutant RyR2 channel dysfunction. In single HL-1 cardiomyocytes, ouabain (EC50 = 14.3nM) perturbed Ca2+ homeostasis in a manner strikingly similar to that determined in cells expressing CPVT-linked R4497C mutation. Moreover, dose-dependent ouabain-evoked Ca2+ dysfunction resulted in a left-shifted caffeine activation profile typical of mutant RyR2 channels. LC/MS/MS analysis determined equivalent levels of ouabain in serum obtained from CPVT- and normal subjects (500-800pM) and exercise stress-testing did not acutely elevate ouabain levels in either cohort. In cells expressing the R4497C mutation, which exhibited basal Ca2+ dysfunction, addition of ouabain at physiologic (1nM) and supra-physiologic (10-100nM) concentrations did not further exacerbate cellular Ca2+ abnormalities. In contrast, the addition of serum obtained from exercised normal and CPVT subjects (100ug protein equivalents; ~2pM ouabain) evoked hallmark mutation-linked RyR2 dysfunction that was not completely abolished by β-blockade. These findings confirm that non-catecholaminergic factors contribute to RyR2 mutant dysfunction, but preclude the possibility that endogenous ouabain is a physiologically relevant trigger of CPVT. However, the hypothesis that endogenous ouabain may induce underlying changes in excitation-contraction coupling that increase the propensity for mutation-linked RyR2 dysfunction is being investigated.

2235-Pos Board B221

The Effect of Adrenergic Stimulation on the Calcium Release Channel

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Adrenergic stimulation of the heart involves phosphorylation of many intracellular Ca^{2+} handling proteins including the ryanodine receptor Ca^{2+} release channels in the SR (RyRs). It is known that RyRs can be phosphorylated at three serine residues at 2808, 2814 and 2030 and that phosphorylation of RyRs via PKA causes an increase in RyR activity *in situ*. However, little is known about how phosphorylation of RyRs in the cell alters their regulation by intracellular Ca^{2+} and Mg^{2+}.

RyRs were isolated from rat hearts, which had been rapidly removed, perfused with Krebs buffer in a Langendorff apparatus. Some hearts were perfused with 1 μM isoproterenol for sufficient time to increase heart rate by >60% for 1 min and others without isoproterenol for 5 mins. Hearts were snap frozen in liquid N_2 to capture their state of phosphorylation, thus allowing RyRs to be phosphorylated by the physiological signalling processes. RyRs were incorporated into artificial planar lipid bilayers and their activity was measured using single channel recording.

The activity of RyRs from isoproterenol stimulated hearts (ISO RyRs, n=25) was 3-fold higher than control RyRs (n=24) at diastolic [Ca^{2+}] (100 nM) but was not significantly different at systolic [Ca^{2+}] (>1 μM). At diastolic [Ca^{2+}], addition of Protein Phosphatase1 (PP1, 5 mins) reduced the activity of ISO RyRs by 98 ± 2.6% (n=4) and control RyRs by 70 ± 20 % (n=4) but this treatment had no effect at systolic [Ca^{2+}]. ISO RyRs displayed a 100-fold channel-to-channel variation in activity which was larger than, and encompassed the range of activity seen either for control RyRs or PP1 treated RyRs. The results indicate that adrenergic stimulation increases RyR2 activity during diastole but not during systole.

2236-Pos Board B222

The Functional Impact of Cis Acting Ryanodine Receptor Type 1 Mutations in a Child with a Fatal Spontaneous MH Event

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Mutations in the RYR1 gene result in MH, a pharmacogenetic disorder of skeletal muscle typically triggered by administration of anesthetics. However, cases of sudden death during exertion, heat challenge, and febrile illness in the absence of triggering drugs have been reported in individuals with RYR1 gene variants. We describe the clinical history and genetic analysis of a child that suffered a fatal, non-anesthetic MH episode associated with febrile illness who was heterozygous for two novel RYR1 variants where one variant, R3983C, occurred *de-novo* and another, D4505H, was inherited. Effects of the two variants on RYR1 sensitivity to activation by caffeine were assessed following expression in RYR1-null myotubes. The single (R3983C and D4505H) and double (R3983C-D4505H) variants were engineered into a full-length rabbit RYR1 cDNA and introduced into RyR1-null myotubes via nuclear microinjection. Effects of the different heterotypic expression conditions (WT+R3983C, WT+D4505H, R3983C+D4505H, and WT+R3983C-D4505H) on RYR1 activation by caffeine were determined in indo-1-loaded myotubes. Compared to WT RYR1 alone (EC_{50} = 1.5 mM), the caffeine sensitivity of RYR1 Ca^{2+} release was only modestly increased following co-expression of either R3983C (EC_{50} = 1.3 mM) or D4505H (EC_{50} = 0.9 mM). Remarkably, co-expression of WT RYR1 with the double mutant in *cis* (R3983C-D4505H) produced a much stronger sensitization of caffeine-induced release (EC_{50} = 0.3) than that observed following co-expression of the two variants on separate subunits (EC_{50} = 0.9). Thus, the R3893C mutation potentiates D4505H-mediated sensitization of caffeine-induced RYR1 Ca^{2+} release when these mutations are in *cis* or present on the same subunit, but not when present on separate subunits. These results indicate that allelic segregation can be a critical, and heretofore unappreciated, pathogenic factor in compound heterozygous MH individuals.

2237-Pos Board B223

GSTM2 C Terminus Reduces Calcium Release Through RyR2 in Spontaneously Contracting and Field Stimulated Ventricular Cardiomyocytes

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The ryanodine receptor (RyR) is an ion channel that releases Ca^{2+} from the sarcoplasmic reticulum and is essential for excitation-contraction coupling and contraction in striated muscle. Previously we reported that the human muscle specific glutathione transferase (GSTM2-2) is an inhibitor of cardiac muscle RyR2 and a weak activator of skeletal muscle RyR1. Single channel experiments and Ca^{2+} release assays using the C-terminal half of GSTM2-2 (GSTM2C) and the mutants, F157A and Y160A in the C terminal domain confirmed the importance of helix 6 in the C-terminal (non-enzymic) fold for inhibition of RyR2. The objective of this study was to determine the effect of GSTM2C on the cardiac myocyte function.

Primary cardiomyocytes were cultured from neonatal rats. Spontaneous or field- stimulated (1Hz with 3V, 2ms pulses) contraction was recorded in control and 15μM GSTM2C-treated cells using a JVC video camera KY/F550 attached to Nikon TE2000-U microscope. Images were analysed using Image Pro plus 6.2 software and percentage cell shortening measured.

Preliminary results showed that spontaneous contraction frequency fell from 42.5/min in the control group to 6.9 /min after GSTM2C treatment ($P<0.001$). The number of spontaneously beating cells fell from 6.6% in the control group to 1.9% after treatment ($P<0.001$). To determine whether the reduced contractions were due to the GSTM2 C terminus affecting action potentials or contraction, shortening was measured. Shortening in spontaneous contraction fell from 7.5 ± 1.0% in control to 2.9 ± 0.6% after GSTM2C treatment ($P<0.001$). Shortening after field stimulation also fell, from 7.3 ± 0.8% before to 4.1 ± 0.6% after, GSTM2C-treatment ($P<0.01$). These results are consistent with GSTM2 C reducing contraction in ventricular cardiomyocytes by reducing Ca^{2+} release through RyR2.

2238-Pos Board B224

In Situ Measurement of RyR2-Calmodulin Binding in Permeabilized Cardiomyocytes

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Calmodulin (CaM) is a Ca-sensing protein and important regulator to RyR2. Defective CaM association with and regulation of RyR2 has been implicated in cardiac hypertrophy, heart failure and Catecholaminergic Polymorphic Ventricular Tachycardia. However, direct measurement of the RyR-CaM binding properties in myocytes is lacking despite its potential clinical importance. This is most likely due to the interference from CaM's multiple binding targets, such as L-type Ca channel, Calcineurin, and CaMKII. Fluorescence Resonance Energy Transfer (FRET) was used to measure the RyR2-specific bound CaM in permeabilized rat and mouse cardiomycytes. FKBP12.6 and CaM bind to RyR2 in close physical proximity so that strong FRET can be detected between fluorescently labeled FKBP12.6 (F-FKBP12.6) and F-CaM. To confirm the specificity of CaM binding to RyR2 and measure the percentage of CaM visible at the Z-line is RyR-bound under physiological conditions, we used mutant RyR2 knock-in mice expressing a triple mutation in the RyR2 ($RyR2^{ADA}$). The mutation prevents CaM binding to RyR2 but should not alter binding of CaM to other targets. Our data showed that 1) the apparent K_d of CaM to RyR in rat cardiac myocytes (at 100 nM [Ca]) was 18.2 ± 2nM (n=XX); 2) heterozygous $RyR2^{ADA/+}$ mutation decreased the maximal binding sites (on RyR2) by 50% without appreciably altering affinity in mouse myocytes (K_d =16.9 ± XX nM in $RyR2^{ADA/+}$ and 13.4 ± XX nM, in WT mice, $p< 0.05$); 3) The total CaM binding visible at the Z-line was reduced by 45%, implying that ~90% of Z-line